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MECHANISMS OF DRUG RESISTANCE IN PLASMODIUM FALCIPARUM

MIDTERM REPORT

DYANN F. WIRTH

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Harvard School of Public Health  
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## 19. ABSTRACT

the *mdr* genes and have completed the sequence, analyzed the expressed mRNA and protein. These genes and their encoded proteins are indeed related to the family of genes known as the ATP-Binding Cassette family and have the highest homology with the *mdr* genes from mouse and human. Thus, the hypothesis was proposed that these genes are involved in drug resistance in *P. falciparum*. Our results implicate a role for the amplification of the *pfmdr1* gene in mefloquine resistance in *P. falciparum*, however, the results remain correlative rather than functional. Our work will now focus on functional analysis of *mdr*-like genes in parasitic protozoa.

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13. ABSTRACT (Maximum 200 words) Drug resistance has emerged as a major problem in the treatment of all microbial agents and in many cancer chemotherapies. This has necessitated the continuous development of new chemotherapeutic agents both for treatment of infectious agents and for cancer chemotherapy. In the case of malaria, drug resistance is an ever present and increasing problem throughout the world with parasites developing resistance to all commonly used antimalarial drugs, even those recently introduced such as mefloquine and halofantrin. Multidrug resistance has emerged as a major problem in the treatment of many cancers and remains a major obstacle to the successful control of certain neoplasias with chemotherapy. It is an emerging problem in parasitic protozoa, and a major question to be investigated in this proposal is the similarity of multidrug resistance in parasites to that found in mammalian cells. In the case of malaria, the similarity in the pharmacological features of the chloroquine resistance in <i>P. falciparum</i> , namely the proposed efflux mechanism and the reversal of resistance by verapamil, desipramine and related compounds led to the proposal that a similar mechanism for drug resistance was operating in <i>P. falciparum</i> . We have identified genes that had sequence and predicted structural similarity to				
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## FOREWORD

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## 5. INTRODUCTION

Drug resistance has emerged as a major problem in the treatment of all microbial agents and in many cancer chemotherapies. This has necessitated the continuous development of new chemotherapeutic agents both for treatment of infectious agents and for cancer chemotherapy. In the case of malaria, drug resistance is an ever present and increasing problem throughout the world with parasites developing resistance to all commonly used antimalarial drugs, even those recently introduced such as mefloquine and halofantrin (Nosten *et al.* 1991; Oduola *et al.* 1987; Shanks *et al.* 1991). Often resistance develops through selection of a mutation in the target enzyme of the drug or in the overexpression of that enzyme. For example, resistance to antifolate drugs is frequently associated with mutations in the dihydrofolate reductase enzyme or in its overexpression. An alternative type of resistance, namely multidrug resistance has emerged as a major problem in the treatment of many cancers and remains a major obstacle to the successful control of certain neoplasias with chemotherapy. It is an emerging problem in parasitic protozoa, and a major question to be investigated in this proposal is the similarity of multidrug resistance in parasites to that found in mammalian cells. This type of resistance is characterized by several unique features and the molecular basis for this resistance is under extensive investigation (Choi *et al.* 1991; Gros *et al.* 1986; Roninson *et al.* 1986; Raymond *et al.* 1990; Udea *et al.* 1987a; 1987b; Guild *et al.* 1988). In the case of multidrug resistance, resistance is observed to a number of structurally distinct drugs each with a different target. Selection of cells resistant to one drug results in the cross resistance to several structurally and functionally unique drugs. The genes associated with this resistance are the multidrug resistance *mdr* genes. The *mdr* gene encode membrane glycoproteins, the P-glycoprotein which mediate the efflux of drugs from the cell. Amplification of the *mdr1* gene in resistant cells results in increased expression of the P-glycoprotein and thus increased efflux of drugs. Thus, the cells are resistant because drug is rapidly removed from the cell before significant toxicity occurs. Use of transfection of the *mdr1* cDNA has demonstrated that overexpression of this gene is sufficient to confer the multidrug resistance phenotype. Drug resistance can be modulated by the use of several compounds including verapamil which appear to inhibit drug efflux. The current hypothesis is that verapamil and related compounds directly bind the P-glycoprotein molecule and block efflux and evidence for direct binding of radiolabelled verapamil to the P-glycoprotein molecule support this hypothesis.

In the case of malaria, the similarity in the pharmacological features of the chloroquine resistance in *P. falciparum*, namely the proposed efflux mechanism and the reversal of resistance by verapamil, desipramine and related compounds led to the proposal that a similar mechanism for drug resistance was operating in *P. falciparum* (Martin *et al.* 1987; Krogstad *et al.* 1992). Both our group under the support of this grant (Wilson *et al.* 1989) and David Kemp's group (Foote *et al.* 1989) identified genes that had sequence and predicted structural similarity to the *mdr* genes and have completed the sequence, analyzed the expressed mRNA and protein. These genes and their encoded proteins are indeed related to the family of genes known as the ATP-Binding Cassette family and have the highest homology with the *mdr* genes from mouse and human. Thus, the hypothesis was proposed that these genes are involved in drug resistance in *P. falciparum*. Further evidence for this proposal was presented by the Foote *et al.* (1990) in

identifying several polymorphism within the *Pfmdr1* gene which appeared to be associated with chloroquine resistance in field isolates. This evidence was in contrast to the analysis by Wellems *et al.* in performing a genetic cross between a chloroquine resistant and chloroquine sensitive cloned parasite (Wellems *et al.* 1990; 1991). In the genetic analysis, both the *pfmdr1* gene and its assorted polymorphism could be dissociated from chloroquine resistance. This was confirmed by a collaboration between our group and the NIH group in which we sequenced the relevant regions of the polymorphism from the resulting progeny (Wilson *et al.*, submitted). Further evidence to refute the association of polymorphism in the *pfmdr1* gene associated with chloroquine resistance was obtained by sequencing recent isolates of drug resistant *P. falciparum* (Wilson *et al.* submitted, see progress report). We have completed this and have demonstrated in 12 new isolates of chloroquine resistant parasites, that the *pfmdr1* gene sequence is identical to that predicted for the chloroquine sensitive phenotype, thus refuting the original Foote *et al.* (1990) claim. Further RFLP analysis of the genetic cross by the Wellems group has determined linkage of the resistant phenotype to a small region of chromosome 7, a location distinct from the known location of either *pfmdr1* or *pfmdr2* (Wellems *et al.* 1991). Thus, the conclusion from this work is that neither the *pfmdr1* or *pfmdr2* gene is directly linked to chloroquine resistance.

The mechanisms of chloroquine resistance remains unknown but progress has recently been reported on a putative target for chloroquine drug action. Slater *et al.* (1992) have reported an enzyme activity, heme polymerase which is hypothesized to be involved in the formation of hemozan pigment and is a method for detoxification of the heme. This enzyme activity in cell extracts is inhibited by chloroquine and related quinolines. Interestingly, the enzyme activity is equally sensitive to chloroquine whether derived from chloroquine sensitive or chloroquine resistant parasites. These results indicate progress towards identifying the primary target of chloroquine action and are consistent with the hypothesized importance of efflux of the chloroquine in drug resistance. The increased efflux phenotype remains associated with chloroquine resistance both in the genetic cross experiments and in new chloroquine resistant field isolates (Krogstad *et al.* 1992; Wellems *et al.* 1990; Watt *et al.* 1990). In addition reversal of chloroquine resistance with verapamil is observed in all chloroquine resistant strains tested (Kyle *et al.* 1990). Thus, the pharmacology of this system remains consistent and has many similarities to the efflux mediated multidrug resistance in mammalian cells. One hypothesis is that chloroquine resistance is mediated by an as yet unidentified efflux pump or, alternatively, that the *pfmdr* genes are involved but that efflux is controlled by the interaction of another gene product with the *pfmdr* genes or encoded proteins.

The role of *pfmdr* genes in other drug resistance mechanisms remains an open and important question. This is particularly the case for mefloquine resistance in Southeast Asia. In our original work, we demonstrated that in a laboratory selected mefloquine resistant cloned parasite, W2mef, the *pfmdr1* gene was amplified when compared to the cloned parent parasite, W2. In subsequent work, Peel *et al.* have demonstrated that under increased mefloquine selection pressure that the *pfmdr1* gene is further amplified approximately 8-10 fold. We also demonstrated an increased expression of *pfmdr1* mRNA in W2mef compared to W2 (Wilson *et al.* 1989; Volkman *et al.* 1992). This work has now been expanded to include several field

isolates of mefloquine resistant parasites and our data suggests that in mefloquine resistant parasites in Southeast Asia, an amplification of the *pfmdr1* gene and an increased expression of mRNA is associated with this resistance (Volkman *et al.* 1992). Further, analysis of the mefloquine resistant strains from Southeast Asia demonstrates that they are cross-resistant, *in vitro*, to other unrelated drugs, similar to the cross-resistance observed in multidrug resistant mammalian cells. Resistance to all drugs can be reversed by penfluoridol and other reversal compounds (Wilson *et al.* submitted). Thus, it appears that mefloquine resistant *P. falciparum* has many of the characteristics in common with multidrug resistant mammalian cells, however, definitive proof of this relationship awaits functional analysis.

The protein encoded by the *pfmdr1* gene has been identified both by our group (Serrano *et al.*, in preparation) and by Cowman (1991) and coworkers using antibody raised against fusion proteins. The P-glycoprotein molecule is 160,000 -170,000 MW and is found associated with membranes in fractionation studies. Cowman finds an association of the protein with the parasite food vacuole and proposes that it is involved with transport in and out of that vacuole. Further investigation of its localization throughout the parasite life cycle and in drug resistant versus drug sensitive parasites is necessary.

While this work was ongoing in *P. falciparum*, both our group and other groups undertook to investigate the potential role of *mdr*-like genes in drug resistance in other parasitic organisms. A similar approach was taken in the identification and cloning of *mdr*-like genes in other parasites. With the collaboration of Dr. Esther Orozco, we cloned two *mdr*-like genes from *Entamoeba histolytica* and demonstrated an association of overexpression of one of these genes with emetine resistance (Samuelson *et al.* 1990; Ayala *et al.* 1990). This work is being continued by Dr. Orozco and Dr. John Samuelson, who was a postdoctoral fellow and is now an independent investigator in the department.

We also initiated studies of the *mdr*-like genes in *Leishmania enriettii* using the same approach of cloning homologous genes using PCR and primers based on the conserved ATP binding sites of the *mdr* gene family (see progress report), and cloned several independent *mdr*-like genes. In collaboration with Dr. Buddy Ullman, Oregon Health Science University, we have identified one of these genes, LEMDR06, as amplified in vinblastine resistant *L. donovani*. In parallel work in *Leishmania tarentole*, Ouelette and Borst identified five *mdr*-like genes, one of which was located on the H-region resistance element (Ouelette *et al.* 1991; 1991a; 1991b). Subsequent work by Callahan and Beverley (1991) and Ouelette *et al.* (1991) have demonstrated this *mdr*-like gene is associated with arsenite resistance in these parasites. The gene identified as associated with arsenite resistance is distinct based on DNA sequence and chromosome location from LEMDR06 which is associated with vinblastine resistance. Thus, in *Leishmania*, there are at least five *mdr*-like genes, two of which are associated with drug resistance. In the case of arsenite resistance, the *pgp* a gene has been transfected into a sensitive strain of parasite and there has been a clear demonstration of the association of this gene with arsenite resistance (Callahan and Beverley 1991). These experiments are underway with the LEMDR06 gene and are described in experimental methods.

The observation that amplification and increased expression of *mdr*-like genes in *Leishmania sp.* is associated with drug resistance in these parasites provides an excellent model system for the analysis of the detailed mechanism of this resistance in a parasitic protozoal system and should provide insights into the analysis of drug resistance in these organisms. The advantage to the *Leishmania* system is the ability to transfect the parasite (Laban *et al.* 1990; Kapler *et al.* 1990) and thus site specific mutagenic analysis of the *mdr*-like genes can be readily undertaken. This has been an extremely powerful approach in mammalian cell systems in the analysis of the *mdr* genes and their functional domains.

It is interesting to note that there are at least five genes in *Leishmania*. This is in contrast to mammalian cells in which there are three genes, two of which are closely related. Thus, one immediate result of the work in *Leishmania* is that we are pursuing potential additional *mdr*-like genes in *P. falciparum* with the notion that although chloroquine resistance is not linked to the two known genes, there may be additional, unidentified *mdr*-like genes, one of which may be involved in chloroquine resistance.

## 6. BODY

Progress has been made on all of the original specific aims of the proposal and brief summaries of the work are included in this section and detailed descriptions in the accompanying appendix material. In addition, primarily because of our development of a transfection system for *Leishmania sp.* thus providing the opportunity for functional analysis of putative *mdr* genes, we initiated studies to identify *mdr*-like genes in *Leishmania* and analyze their role in drug resistance. We plan to use the *mdr* gene system in *Leishmania* as a model system for analysis of these genes in parasitic protozoa. Recent reports of drug resistant leishmaniasis in India may indicate that this is an emerging problem and worthy of investigation in these field isolates.

### Identification of *mdr* genes in *P. falciparum*

These studies were initiated based on the observation that one mechanism of drug resistance in *P. falciparum* may be similar to multi-drug resistance in mammalian cells, namely the mediation of drug efflux by an ATP-dependent efflux pump. A prediction of this model was that the parasite would have *mdr*-like genes and that these would be involved in drug resistance. We used sequences that are conserved in the mammalian P-glycoproteins and several bacterial transport proteins to identify putative *mdr*-like genes in *P. falciparum*. Two primers based on conserved protein sequences shared in the mouse *mdr*, human *mdr* and the bacterial hemolysin B(HlyB) proteins were synthesized. The sense primer was based on a nine amino acid homology found in position 1066-1075 and the antisense primer was based on a seven amino acid homology found in position 1198 to 1204 of the murine *mdr* gene. The codon usage was based on the preferred codon usage for *P. falciparum*.

Two *mdr*-like genes were identified in *P. falciparum* using this approach, *pfmdr1* and *pfmdr2* (Wilson *et al.* 1989). *pfmdr1* was independently identified by the David Kemp's group (Foote *et al.* 1989). Most of our research effort has been focused on the *pfmdr1* gene because

of its association with mefloquine resistance in *P. falciparum* (see below). We have sequenced the entire pfmdr1 open reading frame from a number of independent *P. falciparum* strains (see below, Wilson *et al.* submitted) and the gene encodes a protein with strong homology with mammalian mdr genes both in primary sequence in the highly conserved putative ATP-binding regions and with predicted structural similarity in the transmembrane regions. By Northern analysis (see Figure 1), we have demonstrated that the pfmdr1 gene is expressed at all stages of the asexual parasite life cycle, but that there are different mRNA sizes in rings (single 8.5 kb mRNA) and trophozoite (8.5 kb and 7.5 kb mRNAs) (Volkman *et al.* 1992). These mRNAs are larger than would be predicted based on the size of the open reading frame (4.5 kb) and characterization of the structure of these mRNAs is underway through a combination of reverse transcriptase-PCR analysis, primer extension analysis and nuclease protection analysis. An example of the reverse transcriptase-PCR analysis is shown in Figure 2 which demonstrates that across the coding region of the pfmdr1 gene both mRNAs are colinear with the genomic DNA. In the region 3' to the end of the coding region, there is a divergence between the genomic and mRNA sequence as demonstrated and the basis of this difference is currently being investigated by direct sequence analysis of the PCR products derived from genomic DNA and mRNA.

Sequence analysis of the pfmdr2 gene has identified an open reading frame with significant homology to mammalian mdr genes and bacterial transport genes. Northern analysis of mRNA has demonstrated that the pfmdr2 gene is expressed only in the trophozoite stage in contrast to the pfmdr1 gene which is expressed in all of the asexual stages. Antibody raised against a fusion protein of the predicted extracellular domain of the pfmdr2 protein fused to beta-galactosidase has identified a protein of approximately 100 kd by Western analysis which is expressed in trophozoites. We have detected no difference in levels of expression or pattern of expression of the pfmdr2 gene in drug sensitive versus drug resistant parasites.

### Association of pfmdr1 amplification with mefloquine resistance

Our analysis of mdr genes and drug resistance began with a study of the cloned Indochina strain W2 and its derivative, W2mef which was selected in in vitro culture with increasing concentrations of mefloquine (Oduola *et al.* 1988). When we analyzed the pfmdr1 gene and its expression in these parasites, we discovered that the pfmdr1 gene was amplified 4 fold and there was an increased expression of the pfmdr1 mRNA. There was no apparent change either in gene copy number or expression of pfmdr2 gene.

We wanted to determine if the amplification of the pfmdr1 gene and the increased expression of its mRNA were general properties of mefloquine resistant *P. falciparum*. We therefore extended these studies to mefloquine resistant parasites isolated from patients who failed mefloquine treatment in Eastern Thailand in collaboration with Dr. S. Thaithong. Each parasite strain was tested in vitro for drug sensitivity and then analyzed for pfmdr1 gene copy number and expression.

The results of sensitivity testing are shown in Table I. All of the isolates were tested for sensitivity to chloroquine (CLQ), mefloquine (MFQ), quinine (QUIN),

desethylchloroquine (DCQ), halofantrine (HAL). Isolates PR145, TM327, TM336, TM338, TM342, TM343, TM345, TM346, TM347, TM352 all exhibit a decreased sensitivity to mefloquine when compared to a mefloquine sensitive strain, TM335, isolated from the same geographic region. Interestingly, those parasites resistant to mefloquine show a decreased sensitivity to halofantrine, another aminoquinoline, which has not been used in Thailand. These in vitro results are in agreement with recent reports of clinical resistance of *P. falciparum* to halofantrine in Eastern Thailand (Shanks *et al.* 1992). Additional work needs to be done in order to determine if this is indeed multi-drug resistance occurring in response to treatment with mefloquine.

#### **Amplification and expression of the pfmdr1 gene in mefloquine resistant parasites.**

The gene copy number was determined by quantitative Southern analysis for each of the mefloquine resistant isolates from Thailand. The CSP gene was used as an internal control for each experiment. To confirm that the Thai isolates were distinct, the isolates were analyzed using a repetitive probe which gave a unique fingerprint for each strain. The gene copy number results represent compiled data from three separate experiments (see Table II). In all of the mefloquine resistant Thai isolates tested, the pfmdr1 gene is amplified, while in the mefloquine sensitive isolate TM335 from the same geographic area, there is a single gene copy. These results are consistent with those we originally described using W2 and its mefloquine resistant daughter, W2mef, namely, in mefloquine resistant parasites, there is an increased copy number of the pfmdr1 gene.

We next examined expression of the pfmdr1 gene in a subset of the mefloquine resistant parasites by quantitative Northern and RNA dot blot analysis. The results are presented in Table II. There is an increased expression of pfmdr1 mRNA in mefloquine resistant parasites, either isolated from the field or derived in the laboratory, but not in the mefloquine sensitive strain TM335.

#### **PfMDR-1 sequence**

While this work was ongoing, the Kemp group reported an association of certain polymorphism in the predicted amino acid sequence of the pfmdr1 gene product with chloroquine resistance (Foote *et al.* 1990). Thus, we were interested in determining if a set of polymorphism in the pfmdr1 gene product could be associated with mefloquine resistance. We sequenced the pfmdr1 gene in isolates TM352, TM346 and TM335 to explore the possibility of specific sequences being associated with the drug resistance pattern of these isolates as had been previously suggested for chloroquine. We found a somewhat surprising result, namely the only difference from the original sequence was at bp 1051 (amino acid 184, TAT to TTT, Tyr to Phe). This sequence was shared by all three genes, two from mefloquine resistant parasites TM352 and TM346 and one from a mefloquine sensitive parasite, TM335. Therefore, we concluded that there was no association of this change with mefloquine resistance. However, in comparing our sequence of these genes to those reported by the Kemp group, we noticed that all of our parasite strains are resistant to chloroquine and yet none of them had the sequence

which Kemp's group had predicted as associated with chloroquine resistance. We therefore sequenced the regions of predicted polymorphism in the remaining Thai isolates and found them to be identical to those isolates in which we had sequenced the full-length gene (See Table III). Thus, despite the chloroquine resistant phenotype of these parasites (which we confirmed by our own in vitro testing, see Table I), they did not contain any of the polymorphism identified by the Kemp group as being associated with chloroquine resistance. This result is consistent with the recent work of Wellems *et al.* (1991) which failed to find genetic linkage of *pfmdr1* with chloroquine resistance.

### Identification of *mdr* genes in *Leishmania* sp.

Based on our results with *P. falciparum*, we were interested to determine if there were *mdr*-like genes in other parasitic protozoa and if they had a role in drug resistance. We took a very similar approach, namely the selection of primers based on the conserved ATP-binding site of *mdr* genes followed by PCR amplification of genomic DNA. We cloned and sequenced these PCR products and identified two putative sequences with significant homology to mouse *mdr1*. In separate and parallel experiments, Ouellette and Borst (1991) (Ouellette *et al.* 1991a; 1991b) identified five *mdr*-like genes, at least one of which was contained on the *L. tarentole* amplified H-circle. Subsequent work by Callahan and Beverley (1991) has identified this H-region *mdr*-like gene, named *pgp a* as the genetic element responsible for arsenite resistance in transfection experiments. This gene is distinct based on primary sequence from those genes we identified by PCR amplification using primers based on conserved regions of the ATP-binding site. It appeared that we had identified yet another *mdr*-like gene in *Leishmania*.

The next step was to analyze the expression of this gene in drug resistant parasites. We initiated a collaboration with Dr. Buddy Ullman (see attached letter) at the Oregon Health Sciences University who had selected *L. donovani* parasites with increasing concentrations to vinblastine and isolated the VINB1000 strain which will grow in 1000  $\mu\text{g/ml}$  vinblastine, which is 50 times the concentration which kills sensitive parasites. Our motivation for focusing on these parasites is that vinblastine was one of the drugs most commonly used to select multi-drug resistant mammalian cells which have amplified their *mdr1* genes. In initial Northern and Southern analysis of the VINB1000 compared to the sensitive parental strain DI700, we discovered that sequences homologous to one of our PCR products, LEMR06, were amplified and overexpressed in the resistant parasites. We determined that the gene was amplified 50-100 fold and that the mRNA homologous to the LEMDRR06 gene was expressed at high levels in the VINB1000 cells and not detectable by Northern analysis in sensitive cells. Further characterization of the gene and transcript are underway and the experiments are described in the experimental plan. The VINB1000 cells were tested for cross-resistance to other drugs and were cross-resistant to puromycin and daunomycin. These are drugs which commonly exhibit cross-resistance in the mammalian multidrug resistant cells.

In parallel experiments, we have also begun selection of *L. enriettii* under increasing concentrations of vinblastine and have selected parasites which are able to grow in 120  $\mu\text{g/ml}$

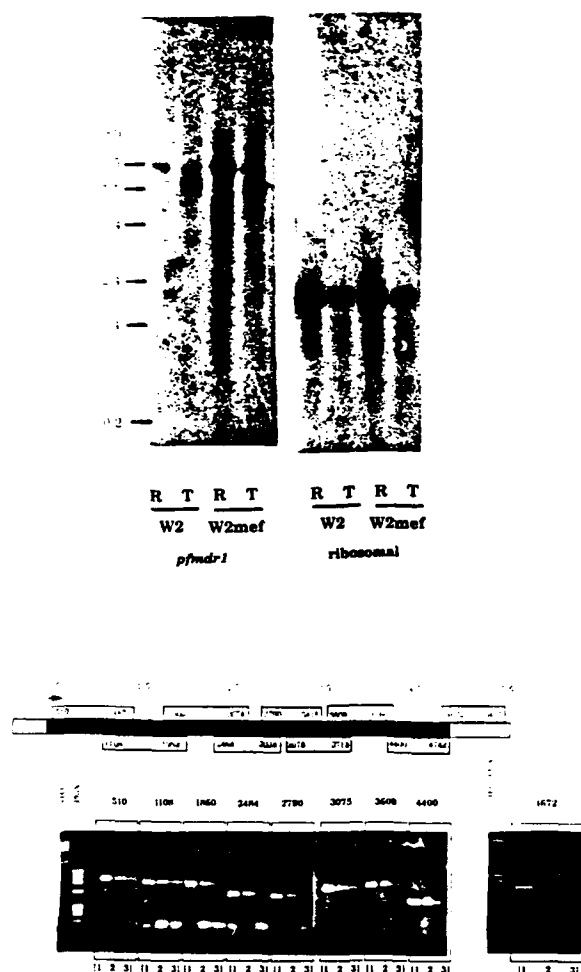
vinblastine or 6 times the concentration which kills sensitive parasites. When these parasites were analyzed by Southern analysis with the LEMDR06 probe (see Figure 3), we discovered that the gene was amplified approximately 10-15 fold when compared to sensitive parental strain. Thus, it appears that the same gene is amplified in vinblastine resistant parasite isolated in an independent experiment with a different parasite strain. Both the VINB1000 and the LE160 vinblastine resistant parasites will be examined in parallel in the subsequent experiments and these experiments will be done in collaboration with Dr. Ullman.

## 7. Conclusions and future experiments

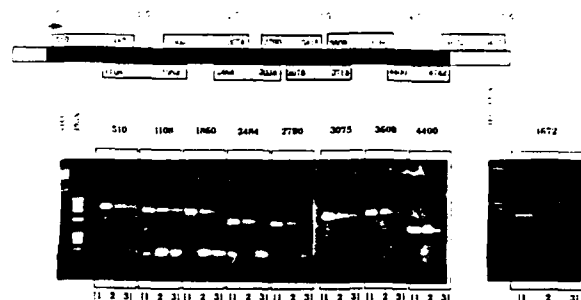
Our results implicate a role for the amplification of the *pfmdr1* gene in mefloquine resistance in *P. falciparum*, however, the results remain correlative rather than functional. Our work during the next grant period will focus on functional analysis of *mdr*-like genes in parasitic protozoa, focusing first on *Leishmania* where *mdr*-like genes have been identified and associated with drug resistance and where we can directly test the function of such genes by genetic transfection analysis. In parallel, we will attempt to develop methods to functionally test the *P. falciparum* *mdr*-like genes, including expression in heterologous systems and development of an endogenous transfection system. We are also aware of the powerful approach of a genetic cross in *P. falciparum* and are actively pursuing potential collaborations. It would be unrealistic to propose such an effort based at Harvard as the resources, both financial and technical to support such a genetic cross are not available. In addition, in my scientific judgement, the development of a transfection system is essential for future work in functional analysis of the *pfmdr* genes and has the added advantage of being useful in many other applications.

The overall goal of this work is to understand the role of *mdr*-like genes in drug resistance in parasitic protozoa. *Mdr*-like genes have been identified in these organisms and there is an association of overexpression of these genes with a multidrug resistant phenotype. However, the definitive proof for such a role lies in the ability to functionally analyze these genes and thus the body of this proposal focuses on the functional analysis of the *mdr*-like genes in parasitic protozoa. The *leishmania* gene will be used as a model system both for developing methodologies and for testing various domains of the gene for functional importance.

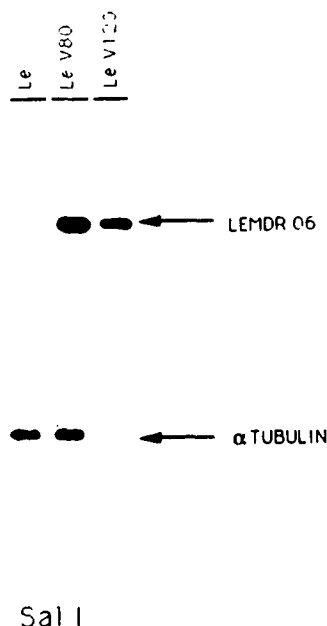
The *Plasmodium falciparum* gene will be tested in heterologous systems and an attempt will be made to develop a homologous transfection system for *P. falciparum*. While this represents a high risk aspect of the project, the value of such a system for the analysis of drug resistance mechanisms and for the analysis of several other important features of parasite biology making, it is a worthwhile undertaking. We have significant expertise in developing transfection systems and have experience in the biology of the parasite. There are other laboratories both in the U.S. and elsewhere who are attempting to develop transfection systems for *P. falciparum*, each with a somewhat different approach. Such multiple efforts greatly enhanced the progress in the field of kinetoplastidae transfection and should one of the other groups be successful, we will use that methodology to investigate the role of *mdr*-like genes in drug resistance in *P. falciparum*. Our interest is in understanding these genes and their function and the development of a transfection system will be an important component in that analysis.



**Figure 1:** Hybridization analysis of stage specific W2 or W2mef RNA. RNA from synchronized cultures was resolved on a formaldehyde agarose gel. The nucleic acid was then transferred to nitrocellulose and hybridized first with the *pfmdr1* probe and then with the ribosomal probe. RNA samples from both ring (R) and trophozoite (T) stage cultures of W2 and W2mef.



**Figure 2:** Structural analysis of *pfmdr1* mRNA. Total W2mef RNA was reverse transcribed using random hexamers and PCR analysis was performed using oligonucleotides, whose 5' base pair position is indicated by the number across the coding region, represented by the black box. The expected products are indicated by the stippled boxes, except for the 3' polymorphic region which is shown as a white box. PCR products from either W2mef DNA, W2mef RNA + RT, or W2mef RNA - RT as target were run on agarose gels and stained with ethidium bromide. The results of this analysis are shown with the forward primer number listed above the set of three lanes.



**Figure 3:** *L. enriettii* cells were grown in Schneider medium supplemented with 15% FCS. Three days old culture was harvested and resuspended in NET (0.1 M NaCl, 0.01 M EDTA pH 8.0, 0.01 M Tris pH 8.0). The cell suspension was then treated with 5mg RNase A and 5mg Proteinase K, followed by phenol, chloroform extraction and precipitation. 5 µg of genomic DNA was electrophoresed on a 0.8% agarose gel and blotted onto nylon filter paper. About  $5 \times 10^6$  cpm was used for hybridization. Stringent washing condition was used (2x SSC, 0.5% SDS, RT for 30 min, 0.1x SSC, 0.5% SDS, 65°C for 30 min). Autoradiography was done at -70°C.

### Figure 4

## LEMDR 06

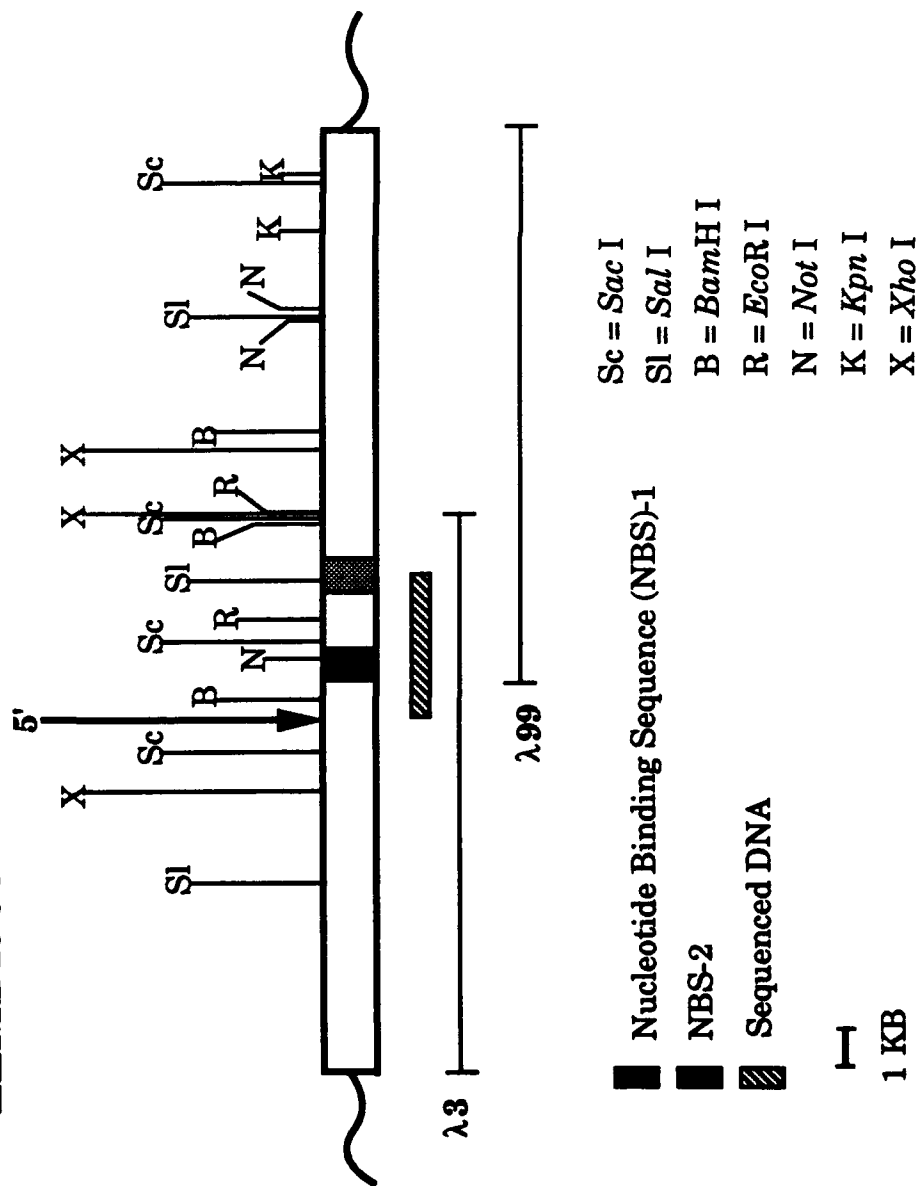


TABLE I

DRUG SENSITIVITIES OF <i>P. FALCIPARUM</i> THAI ISOLATES (ng/ml)						
Strain	CLQ	MFQ	QUIN	DCQ	HAL	
PR145	35	10	90	168	2.1	
TM327	42	16	59	158	2.7	
TM336	38	15	87	182	2.8	
TM338	29	9	53	170	1.9	
TM342	46	8	80	200	2.1	
TM343	39	14	99	290	3.3	
TM345	65	9	128	62	2.3	
TM346	31	20	114	135	3.7	
TM347	23	17	62	84	3.6	
TM352	27	22	102	117	3.0	
GA3	41	11	49	161	2.0	
TM335	68	6	65	267	.61	
Sensitive	<10	<8	<25	<50	<1.5	

Results are given as IC<sub>50</sub>'s from a standard 48 hour hypoxanthine uptake assay (Desjardins *et al.* 1979). Abbreviations: CLQ = chloroquine; MFQ = mefloquine; QUIN = quinine; DCQ = desethylchloroquine; HAL = halofantrine.

TABLE II

QUANTIFICATION OF MFQS OR MFQR ISOLATES FROM THAILAND			
Strain	MFQ (ng/ml)	Gene Copy Number	Total RNA
W2	S (2)	1	1
W2mef	R (15-20)	3	2.4
TM335	S (6)	1	1
PR145	R (10)	4	3
TM327	R (16)	2	1.8
TM336	R (15)	2	3.3
TM338	R (9)	>2	2.2
TM346	R (20)	>2	2
TM352	R (22)	>3	2.6

Total RNA from some recent isolates from Thailand along with the W2 strain were analyzed by slot hybridization. Median inhibitory concentrations for MFQ and *pfmdr1* gene copy number are shown. Values are expressed as the ratio of *pfmdr1* to ribosomal signal, normalized to W2.

TABLE III

DNA SEQUENCE ANALYSIS						
	AA*	86	184	1034	1042	1246
	nt*	754	1051	3598	3622	4234
ISOLATE						
Sensitive#		AAT	TAT	AGT	AAT	GAT
R:SE Asia#		TAT	TAT	AGT	AAT	GAT
R:S Amer#		AAT	(TTT)	TGT	GAT	TAT
PR145		AAT	TTT	AGT	AAT	GAT
TM327		"	"	"	"	"
TM335		"	"	"	"	"
TM336		"	"	"	"	"
TM338		"	"	"	"	"
TM342		"	"	"	"	"
TM343		"	"	"	"	"
TM345		"	"	"	"	"
TM346		"	"	"	"	"
TM347		"	"	"	"	"
TM352		"	"	"	"	"
GA3/GH2		AAT	TAT	AGT	AAT	GAT

Complete sequence of *pfmdr1* from four isolates (TM352, TM346, TM335 and GA3) was determined. The difference at amino acid #184 was the only difference seen from the previously published sequence. The sequence is shown for the sites identified by Foote *et al.* 1990 as "alleles" associated with chloroquine resistance.

\*Amino acid numbers and nucleotide numbers of *pfmdr1* are as determined in Foote *et al.* 1989.

#Sensitive is the wild-type genotype; R:SE Asia and R:S Amer are resistant Southeast Asian and South American genotypes as described in Foote *et al.* 1990.

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